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# Potentiality of Improving or Suppressing Tyrosinase Inhibitory Activity by Media Composition for the Marine Fungus *Aspergillus unguis* SPMD-EGY

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**ABSTRACT**

*Marine-derived fungi have proven to be a prolific source of secondary metabolites with interesting structures and biological activities. Tyrosinase is a key enzyme for melanin biosynthesis and is associated with melanin hyper-pigmentation and melanoma. Various culture conditions are able to produce variable bioactive secondary metabolites. Three different broth media; Potato dextrose (PD), PYMG and Czapek-Dox, in addition to one solid medium (Rice medium) were used for the cultivation of the fungus and tested for their potentiality to improve Tyrosinase inhibitory activity. The mycelia static extract of PD medium showed strong inhibitory activity (48%), followed by moderate activity for the culture static extracts of DOX and PYMG media (41 and 40%), comparing to the drug activity (vitamin C, 60%). The mycelia and culture shake of the three media, in addition to Rice solid medium extracts showed low activity. The big surprise is for PYMG culture static, mycelia and culture shake extracts, where by using distilled water instead of sea water and the reduction in glucose concentration (from 10 to 5 %) led to significant sudden increase of tyrosinase inhibitory activity from (0 to 40%). GC/MS analysis of PD extract revealed the presence of 22 compounds. The presence of benzoic acid, 2,4-dihydroxybenzoic acid, caffeic acid, benzeneacetic acid-α-4-dihydroxy, benzeneacetic acid-2-hydroxy, benzenepropanoic acid-α-hydroxy improved the level of the tyrosinase inhibitory activity. PYMG culture static extract contained 2,2'-dihydroxy-chalcone (conc. 34%) and 2,4,4'-trihydroxy-chalcone (conc. 3%), which have tyrosinase inhibitory activity.*

**Keywords:** Corals, *Aspergillus unguis* SPMD-EGY, Media compositions, Tyrosinase inhibitors, GC/MS analysis

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## INTRODUCTION

Marine-derived fungi have proven to be a prolific source of secondary metabolites with interesting structural properties and biological activities [1]. Marine fungi are potential producers of bioactive compounds that may have pharmacological and medicinal applications. Immense efforts have been made to investigate marine-derived compounds. Unique and stressful marine habitats have profound effects on fungal biological activity [2]. The success of fungal metabolites can be attributed to many factors, like the advances in the industrial production of biotechnological metabolites and the possibility of working with techniques such as the "OSMAC"(one strain-many compounds) [3]. Many marine derived fungal biological activities have been detected, including antioxidant, cytotoxic, antitumor, antifungal or anti-inflammatory activities [4].

Melanin is the major cellular component responsible for developing skin color in human beings. It is a complex; heterogeneous polyphenol-like biopolymer structure and its color vary from yellow to black. Melanin is secreted by melanocyte cells in the basal layer of the dermis [5]. Normal melanin pigmentation is able to shield from UV radiation, While, the abnormal pigmentation, such as senile lentigines, freckles, melasma, and other forms of melanin hyperpigmentation, causes serious Health problems [6]. The oxidative reactions of tyrosine catalyzed by tyrosinase enzyme contribute to melanin biosynthesis [7]. Tyrosinase (monophenol monooxygenase EC 1.14.14.1) is a binuclear copper enzyme, catalyzes two distinct reactions of melanin biosynthesis. Tyrosinase contains two copper ions that coordinate with histidine residues in the active site. The two copper ions are of critical importance for the catalytic activity of this enzyme [8]. Since tyrosinase-catalyzed reactions are highly associated with hyperpigmentation, the discovery of new natural tyrosinase inhibitors are of great importance in cosmetic and medicinal products for the prevention of pigmentation disorders [9]. Recently, significant efforts have been made to search for the tyrosinase inhibitors with copper chelator ability as whitening and anti-hyperpigment agents [10]. Many tyrosinase inhibitors, such as hydroquinone, kojic acid, azelaic acid, electron-rich phenols and arbutin have been tested in pharmaceuticals and cosmetics for their capability of preventing overproduction of melanin [11]. The inhibition of tyrosinase, decreasing of melanocyte metabolism and avoiding of UV exposure are ways to reduce melanin synthesis. Tyrosinase inhibitors are of great importance in treatment for pigmentation and developed as cosmetically skin-whitening agents [12].

This work aimed to screen the effect of the fungus *Aspergillus unguis* SPMD-EGY secondary metabolites through using different media to find out the effective one as tyrosinase inhibitor.

## MATERIALS AND METHODS

### Fungal isolation

The internal part of S*inularia* sp. (a fresh soft coral) was cut to minute samples of about $1cm^3$. These samples were immersed in sterile sea water three times followed by ethyl alcohol (70%) for about 30sec for sterilization. The sterilized samples were used, as they are or after homogenization under sterile conditions and dilution up to $10^{-6}$, to inoculate a fungal isolation medium [13].The inoculated plates were incubated at $30^oC$ until the appearance of single pure colonies which were then picked and maintained on potato dextrose agar medium (PDA) and kept at $4^oC$ and further use [3].

### Molecular identification

Molecular identification of fungal strain has been established by DNA extraction, PCR and sequencing as previously mentioned [13].

### Selection of the culture media

The isolate *Aspergillus ungius* SPMD-EGY was grown on different culture media:

**A.** Potato dextrose broth (PD) (g/l): 200g of peeled potato and glucose (20).

**B.** Peptone yeast extract malt extract glucose medium [PYMG] (g/l): yeast extract (3),malt extract (3), peptone (5) and glucose (5);

**C.** Czapek's Dox broth (DOX) (g/l): sucrose (30), sodium nitrate (3), dipotassium phosphate (1), potassium chloride (0.5), magnesium sulphate (0.5) and ferrous sulphate (0.1). All media were dissolved in distilled Water.

**D.** Rice solid medium (RS) of the following ingredients: rice (100g) and distilled water 100 ml in 1l-Erlenmeyer flasks.

### Extraction of secondary metabolites

The culture supernatant was extracted with ethyl acetate (3x or till exhaustion) and then evaporated under vacuum. On the other hand the fungal mycelia were first extracted using acetone and evaporated till dryness. The residual part was re-extracted using small volume of ethyl acetate [3], while for the rice solid medium, it was directly extracted with ethyl acetate, followed by filtration and evaporation [14].

### Mushroom Tyrosinase inhibitory Activity

To evaluate the inhibitory action of supernatant and mycelia extracts on tyrosinase; tyrosinase isolated from mushrooms was utilized as described previously with a minor modification [15, 16]. In brief, 140 µl 50 mM phosphate buffer (PH 6.8), 10 µl of extract (1mg/ml, dissolved in MeOH), 40 µl of 1.5mM L-tyrosine solution and 20 µl of Mushroom tyrosinase (1500 U/ml) were added to a 96 well microplate. The assay mixture was incubated at

25 °C for 30 min. Following incubation, the amount of dopachrome produced in the reaction mixture was determined spectrophotometrically at 492 nm in a microplate reader.

**GC/MS analyses**

A Finnigan MAT SSQ 7000 mass spectrometer was coupled with a Varian 3400 gas chromatograph. DB-5 column, 30 m x 0.32 mm (internal diameter) , was employed with helium as carrier gas (He pressure, 20 Mpa/cm$^2$), injector temperature, 310ºC; GC temperature program, 85 - 310$^o$C at 3 $^o$C/ min (10 min. initial hold).The mass spectra were recorded in electron ionization (EI) mode at 70 eV. The scan repetition rate was 0.5 s over a mass range of 39 - 650 atomic mass units (amu).

***Sample preparation for GC/MS analyses***

1mg of the dried extract was prepared for chromatography by derivatization for 30 min at 85°C with 15 µl pyridine + 20 µl N,O, bis-(trimethylsilyl)trifluoroacetamide (BSTFA) and analyzed by GC/MS [17].

***Identification of compounds***

The identification was accomplished using computer search user-generated reference libraries, incorporating mass spectra. Peaks were examined by single-ion chromatographic reconstruction to confirm their homogeneity. In some cases, when identical spectra have not been found, only the structural type of the corresponding component was proposed on the bases of its mass spectral fragmentation. Reference compounds were co-chromatographed when possible to confirm GC retention times.

## RESULTS AND DISCUSSION

**Fungal identification**

The fungus was previously identified [13] using the molecular protocol (18SrRNA) as *Aspergillus unguis* SPMD-EGY and was deposited in the Gene Bank with the accession Number KM203833 and appears in the PubMed through the following link (site) (http://www.ncbi.nlm.nih.gov/nuccore/KM203833).

**Effect of culture composition on Tyrosinase inhibition**

The promising fungal strain (SPMD) was isolated and molecularly identified as *Aspergillus unguis* SPMD-EGY[13].

The isolated fungus was tentatively identified in previous work as "*Emericella unguis* 8429", where it showed moderate inhibitory activity against acetylcholinesterase and α-glucosidase enzymes [3, 18], while had no effect against tyrosinase enzyme [unpublished data]. Acetylcholinesterase and α-glucosidase inhibition results prompted us to extend our work by studying the effect of different parameters that could be useful in improving these biological activities.

Media composition is one important parameter that had been proven to affect the biological activity of 2ry metabolites. Three different broth media; Potato dextrose broth (PD), PYMG and Czapek-Dox (DOX) media, in addition to one solid medium (Rice medium) were used; remarkable data were obtained improving the inhibitory activities against acetylcholinesterase and α-glucosidase enzymes [3, 13].

Investigating the effect of extracted 2ry metabolites from these media against tyrosinase enzyme revealed that, the mycelia static extract of PD medium (Figure 1a), showed strong inhibitory activity (48.4%), followed by moderate activity for culture static extracts of DOX and PYMG media (41 and 40%, Figure 1c & 1b), comparing to the drug activity (vitamin C, 60%). The mycelia and culture shake, in addition to culture static of PD medium showed low inhibitory activity about 18% (Figure 1a). The PYMG medium mycelia and culture shake showed moderate inhibitory activity (34 & 30%, Figure 1b), while its mycelia static exhibited no activity. The mycelia and culture shake of DOX medium showed low inhibitory activity (19 & 15%, Figure 1c), while mycelia static extract had no activity.

So, it is clear that from the three mycelia static of the three different media; PD is the only one showed inhibitory activity against tyrosinase enzyme. Rice solid medium extract showed low inhibitory activity (26%, Figure 1d).From these findings, it was clear that the inhibitory effect of fungal extract depends on the type of culture medium used.

The big surprise is the sudden increase for PYMG culture static, mycelia and culture shake extracts from 0.0 % for all to 40, 34 and 30 % respectively, (Figure 2; B2& B1), while mycelia static still had no effect in both media. It was clearly observed that using distilled water instead of sea water affects the biological activity of the fungus. This could be attributed to the stress exerted by sea water that affects the endocellular 2ry metabolites formation and consequently the activity. Also, the reduction in glucose concentration (from 10 to 5 %) could be another reason for the significant presence and sudden increase of tyrosinase inhibitory activity from (0 to 40%, Figure 2; B2& B1). From this suggestion; may both endocellular and exocellular 2ry metabolites have been affected for the mycelia and culture shake, while for culture static, exocellular 2ry metabolites have been only affected.

Our data were in agreement with the optimization study of antimicrobial metabolite production from *Aspergillus terreus*, where salinity is one of the parameters tested and expressed as NaCl. They found that low salinity (5g/l NaCl) exhibited excellent growth and bioactive metabolites [19, 20].

Glucose, the best carbon source for growth, depressed the biosynthesis of several metabolites like actinomycin and cephalosporin and not affects the production of aminoglucosides and chloramphenicol [21]. Hutter reported that the addition of glucose resulted in high fungal growth, but the production of bioactive metabolites were decrease [22]. Glucose has a suppression effect of bioactive secondary metabolites in many fermentation processes [23].

It has been found that the fermentation culture medium is an essential criterion for the production of enough extract for maximum production of tyrosinase inhibitor. Vasantha *et al.*, isolated 26 fungal strains (S1-S26) using potato dextrose agar medium supplemented. Strain number S16 exhibited the highest inhibition percent (80%). This strain was identified as *Aspergillus niger* and the strain was cultivated on different media including: potato dextrose broth, Czapek Dox broth, Sabaurod's broth, Glycerol broth, Sucrose broth, yeast extract malt extract broth, nutrient broth, oat meal broth and malt extract broth. Potato dextrose broth showed the highest tyrosinase inhibitory effect (80%) followed by glycerol broth (45%), oat meal broth (33%) and Czapek Dox broth medium (32%) [24]. Tyrosinase inhibitors were classified into five classes i.e., polyphenols, benzoate derivatives, long chain lipids, benzaldhyde and steroids. Kojic acid is commonly known as tyrosinase inhibitor and isolated from fungi as *Aspergillus ni*ger [25].

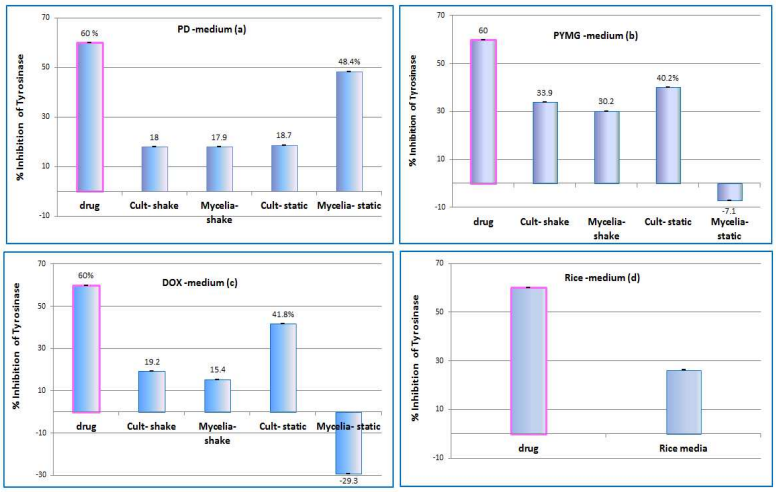


**Figure 1: % Tyrosinase inhibitory activity of 2ry metabolites extracts from culture and mycelia (shake and static) of different media. Values are expressed as mean ±SD, n = 3 (200 µg/ml for all tested extracts and drug; Vit. C)**

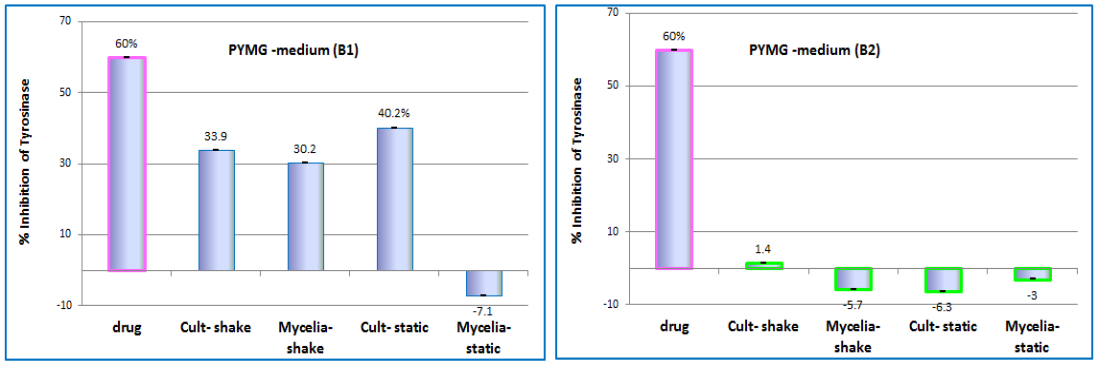


**Figure 2: Demonstrative comparison between PYMG media (B1, 5% glucose, distilled water) and (B2, 10% glucose, sea water), for their 2ry metabolites extracts as Tyrosinase inhibitors**

## GC/MS analysis

GC/MS analysis of the highly active mycelia static extract of PD medium of the identified fungus (*Aspergillus unguis* SPMD-EGY) isolated from the soft coral *Sinularia* sp. revealed the identification of 22 compounds. The major compounds identified were: butanoic acid-2-methyl, butanoic acid-4-hydroxy, 3-pyridinecarboxylic acid, propanoic acid-2,3-dihydroxy, 2-isopropyl-2-hydroxy succinic acid, benzoic acid, caffeic acid, 2,4-dihydroxybenzoic acid, benzeneacetic acid-α,4-dihydroxy, benzene-acetic acid-2-hydroxy and benzene-propanoic acid-α-hydroxy (Table 1).

The presence of benzoic acid, 2,4-dihydroxybenzoic acid, caffeic acid, benzene-acetic acid-α,4-dihydroxy, benzeneacetic acid-2-hydroxy, benzenepropanoic acid-α-hydroxy in mycelia static extract of PD sample improved the level of the tyrosinase inhibitory activity (Table 1, Figures 1a & 3). The high percent of dodecanoic acid (C12:0) (7%) probably affected to some extent the tyrosinase inhibitory activity to be not more than 48%. Our data were in agreement with previous studies, where free fatty acids have been proven to show remarkable regulatory effects on melanogenesis, *i.e.* unsaturated fatty acids such as oleic acid (C18:1), linoleic acid (C18:2), and α-linolenic acid (C18:3) decrease melanin synthesis and tyrosinase activity, whereas saturated fatty acids such as palmitic acid (C16:0) and stearic acid (C18:0) increase melanin synthesis and tyrosinase activity [26]. These data supported our results, where the previously identified [13] fatty acids; hexadecanoic acid (C16:0, conc.,18%) in PYMG sample (B2, culture static), could be the cause of suppressing the tyrosinase inhibitory activity (Figure 2; B2), despite of the minor presence of the unsaturated fatty acids: 9,12-octadecadienoic acid (C18:2), 9-octadecenoic acid (C18:1), and 11-octadecenoic acid (C18:1).

The GC/MS analysis revealed the presence of several bioactive compounds that are reported to display tyrosinase inhibitory activity (Figure 3).The inhibition of tyrosinase by benzoic acid is a reversible reaction with remaining enzyme activity. Benzoic acid was found to be a noncompetitive inhibitor for the oxidation of L-DOPA [27].

Caffeic acid exhibited potent anti-tyrosinase activity. It exhibited noncompetitive inhibition against oxidation of L-DOPA by mushroom tyrosinase [28].

The inhibition kinetics of 2-phenylethanol, 2-phenylacetaldehyde and 2-phenylacetic acid on the enzyme activity of mushroom tyrosinase have been investigated. The results showed that these aromatic compounds can lead to reversible inhibition of the enzyme. The inhibition constants have been determined and the inhibiting ability was: 2-phenylacetaldehyde>2-phenylacetic acid>2-phenylethanol, indicating that the functional group on the benzene ring group played an important role in the inhibition of the enzyme [ 29]. 2,4-dihydroxybenzoic acid showed weak tyrosinase inhibitory activity with $IC_{50}$ values over 200 µM [30]. Dihydroxybenzoic acids (DBA), such as 3,4-DBA, 3,5-DBA, and 2,4-DBA (at all concentrations tested) inhibited the rate of DL-DOPA oxidation to dopachrome by mushroom tyrosinase [31].

All the above mentioned data are in agreement with our results and the identified compounds (Table 1, Figures 1&3).

The demonstrative Figure 2 (2B1) showed that PYMG culture static extract showed 40% inhibitory activity, while PYMG culture static extract (Figures 2B2) had no activity. Previous study [3], showed that PYMG culture static extract (2B1) contained 2,2'-dihydroxy-chalcone (conc. 34%) and 2,4,4'-trihydroxy-chalcone (conc. 3%). These chalcones were reported to have tyrosinase inhibitory activity, where Gutierrez *et al.* mentioned that the chalcones: 4-hydroxychalcone, 4'-hydroxychalcone, 2'-hydroxy- chalcone, 2',4'-dihydroxychalcone, 2',4-dihydroxychalcone, 2',4',4-trihydroxy- chalcone and 2',4',3,4-tetrahydroxychalcone were tested as inhibitors of tyrosinase activity, showing that the most important factor in their efficacy is the location of the hydroxyl groups on both aromatic rings, with a significant preference to a 4-substituted B ring, rather than a substituted A ring. Neither the number of hydroxyls nor the presence of a catechol moiety on ring B correlated with the increasing tyrosinase inhibition potency [32].

**Table 1: Chemical composition assessed by GC/MS analysis for the PD medium (mycelia static extract) of the fungus *Aspergillus unguis* SPMD-EGY**

| No | Compound | PD(%)[a] |
|---|---|---|
| | ***Nitrogenous compounds*** | |
| 1 | N-l-valyl-l-valinate | 0.12 |
| 2 | 3-Pyridine-carboxylic acid | 0.1 |
| 3 | 1-Imino6(4-methylphenyl)3-oxo-2,5dipheny-l1,2,3,5,6-pentahydro-imidazo[1,5a]pyrazine-8-carboxamide | 2.5 |
| | | |
| | ***Fatty acids / esters*** | |
| 4 | Butanoic acid-2-methyl | 0.78 |
| 5 | Butanoic acid-4-hydroxy | 0.05 |
| 6 | Propanoic acid, 2,3-dihydroxy | 0.22 |
| 7 | 2-isopropyl-2-hydroxy succinic acid | 0.04 |
| 8 | Dodecanoic acid (C12:0) | **7.13** |
| 9 | Hexadecanoic acid (C16:0) | 1.63 |
| 10 | 9-Octadecenoic acid (C18:1) | 1.11 |
| 11 | Octadecanoic acid (C18:0) | 0.41 |
| 12 | Hexadecanoic acid-2,3-dihydroxy propyl ester | 0.16 |
| 13 | Octadecanoic acid- 2,3-dihydroxy- propyl ester | 0.11 |
| | | |
| | ***Phenolic acids*** | |
| 14 | Benzoic acid | 0.14 |
| 15 | Benzeneacetic acid-α-4-dihydroxy | 0.13 |
| 16 | Benzeneacetic acid-2-hydroxy | 0.17 |
| 17 | Benzenepropanoic acid-α-hydroxy | 0.09 |
| 18 | 2,4-Dihydroxybenzoic acid | **2.65** |
| 19 | Caffeic acid | **0.64** |
| | ***Others*** | |
| 20 | Erythrose | 0.07 |
| 21 | D-Galactonic acid-α-lactone | 0.11 |
| 22 | Diisooctyl phthalate | 0.16 |

*a = The ion current generated depends on the characteristics of the compound concerned and it is not a true quantitation.*

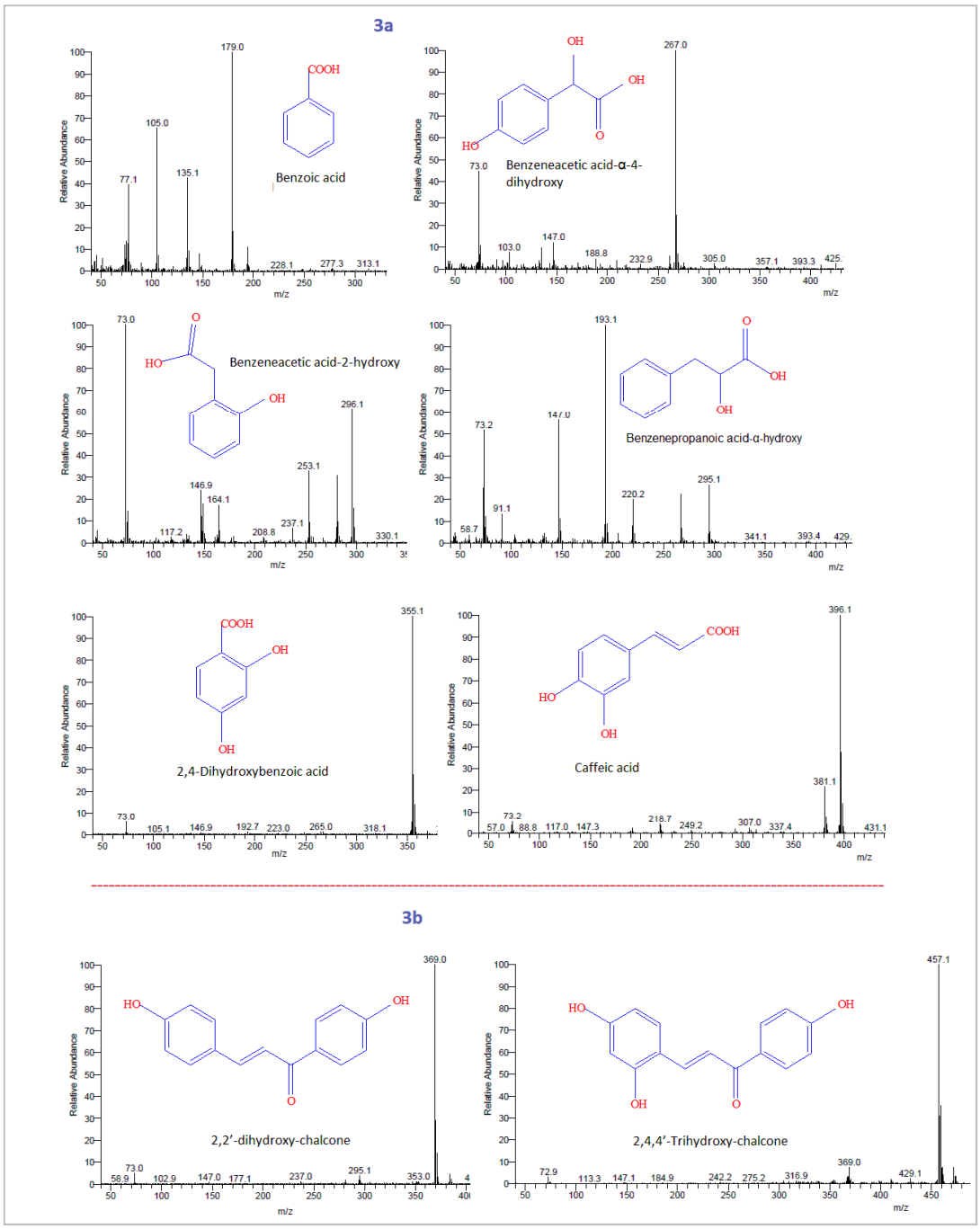


**Figure 3: Structure of some compounds identified In [PD, 3a] and [PYMG, 3b] extracts which were known to have tyrosinase inhibitory activity**

## CONCLUSION

It could be concluded that, the secondary metabolites of the isolated marine fungus ***Aspergillus unguis*** **SPMD-EGY** showed tyrosinase inhibitory activity, our study provides (for the first time) primary evidence suggesting that these secondary metabolites in further in-vivo studies could play an important role as tyrosinase inhibitor.

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